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ANION AND CATION REQUIREMENTS FOR GLUCOSE AND METHIONINE ACCUMULATION BY *HYMENOLEPIS DIMINUTA* (CESTODA)[1]

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The Na^+- or ion-gradient hypothesis (see Schultz and Curran, 1970, for a review) postulates that the energy necessary for the intracellular accumulation of solutes against a concentration difference is obtained, ultimately, from the maintenance of a Na^+, and possibly K^+, difference across the cell membrane. In addition to evidence that such a mechanism is functional in glucose and amino acid influx and accumulation in some vertebrate tissues (Schultz and Curran, 1970), there is ample evidence that such systems also function in glucose influx in tapeworms. Glucose influxes in *Taenia* (*Hydatigera*) *taeniaeformis* larvae and adults, *Hymenolepis diminuta* larvae and adults, *Calliobothrium verticillatum* adults, and *Taenia crassiceps* larvae are Na^+-sensitive (von Brand and Gibbs, 1966; Arme, Middleton and Scott, 1973; Dike and Read, 1971; Fisher and Read, 1971; Pappas, Uglem and Read, 1973a, respectively). Glucose and Na^+ movements into *C. verticillatum* are coupled, and the kinetics of glucose and Na^+ fluxes in this species suggest that the mechanisms of Na^+-coupled glucose influx in tapeworms and vertebrate tissues are generally similar (Pappas and Read, 1972).

On the other hand, the influxes of phenylalanine and methionine in *T. crassiceps* larvae and methionine in *H. diminuta* are insensitive to the external Na^+ concentration (Pappas, Uglem and Read, 1973b, Read, Rothman and Simmons, 1963, respectively), although these amino acids are accumulated by the worms in the presence of Na^+. Moreover, *T. crassiceps* larvae accumulate phenylalanine and methionine in Na^+-free media (Pappas *et al.*, 1973b). This raises the question of whether ions and/or ion differences (gradients) are related to amino acid influx and accumulation in tapeworms. Also, although glucose influx in several tapeworm species is known to be Na^+-sensitive and/or Na^+-coupled, it is not known whether glucose accumulation is Na^+-dependent. This paper reports an experimental examination of ion requirements for glucose and amino acid influx and accumulation in the tapeworm, *Hymenolepis diminuta*.

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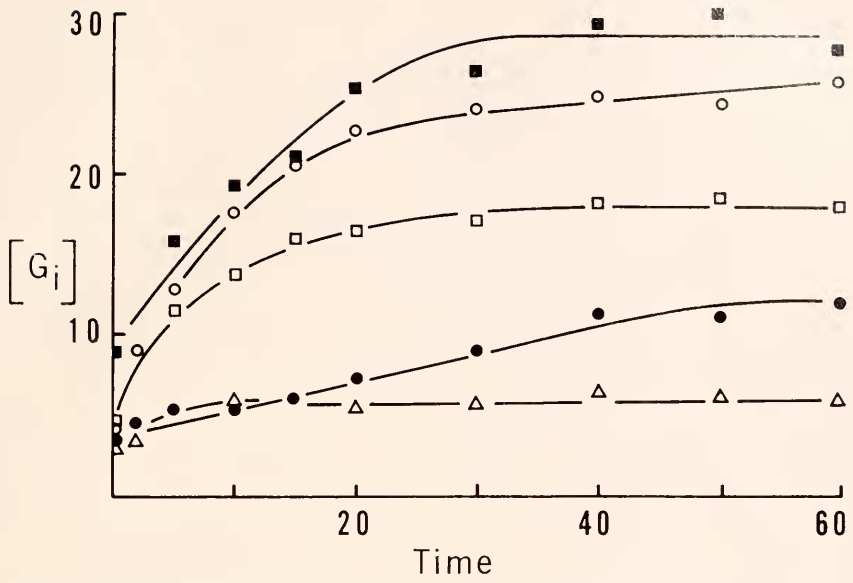

FIGURE 1. The internal glucose concentration ($[G_i]$, μmoles glucose/ml worm water) of *Hymenolepis diminuta* incubated in 5 mM glucose in KRT, or various ion-substituted KRT solutions, versus time of incubation (min). Closed circles equal control (worms incubated in KRT only); open circles equal worms incubated in glucose in KRT; open triangles equal worms incubated in glucose in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in glucose in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3COO^- as the replacement anion. Each point is the mean of 3 replicates.

MATERIALS AND METHODS

Specimens of *Hymenolepis diminuta* from 10-day-old, 30-worm infections of young male Sprague-Dawley rats (Holtzman Co.) were used in all experiments. After removal from the host, worms were washed in several changes of a Krebs-Ringer solution containing 25 mM tris(hydroxymethyl)-aminomethane-maleate buffer at pH 7.4 (KRT of Read *et al.*, 1963), and randomized into groups of 5 worms (approximately 200 mg wet wt). Prior to incubation, each group of worms was preincubated in 10 ml of fresh KRT (or the appropriate ion-substituted KRT) for 30 min. All preincubations and incubations were carried out at 37° C.

In L-methionine and D-glucose accumulation studies, the worms were removed from the preincubation medium, blotted dry, weighed (± 0.5 mg), and incubated in 10 ml of KRT (or the appropriate ion-substituted KRT) containing either L-methionine or D-glucose. After intervals of time indicated in various experiments, the worms were removed, rinsed rapidly in 3 changes of KRT, blotted dry, weighed (± 0.5 mg), and extracted overnight in 70% ethanol. Glucose in the ethanol extracts and incubation media were determined using the glucose oxidase method (Glucostat Special, Worthington Biochemicals), and methionine in the ethanol extracts (following partitioning against acidified chloroform) and incubation media was determined by the method of Horn, Jones and Blum (1946). Glu-

cose and methionine concentrations in the worms were calculated in terms of μmoles solutes/ml worm water at the end of the incubation period. Following ethanol extraction, the worms were digested in 30% KOH and the 50% ethanol precipitable carbohydrate (glycogen) was determined by the method of Dubois, Giles, Hamilton, Rebers and Smith (1956).

To measure short term glucose and methionine influx rates, groups of worms were incubated in 5 ml of KRT (or the appropriate ion-substituted KRT) containing 0.25 μCi/ml of D-glucose-^{14}C(U) or L-methionine-^{14}C(methyl) (New England Nuclear). After a 2 min incubation, the worms were removed, rinsed rapidly in 3 changes of KRT, blotted dry and extracted overnight in 2 ml of 70% ethanol. Radioactivity in 1 ml aliquots of the ethanol extracts was determined with a Nuclear-Chicago gas-flow counter. The worms were dried overnight at 95° C and weighed.

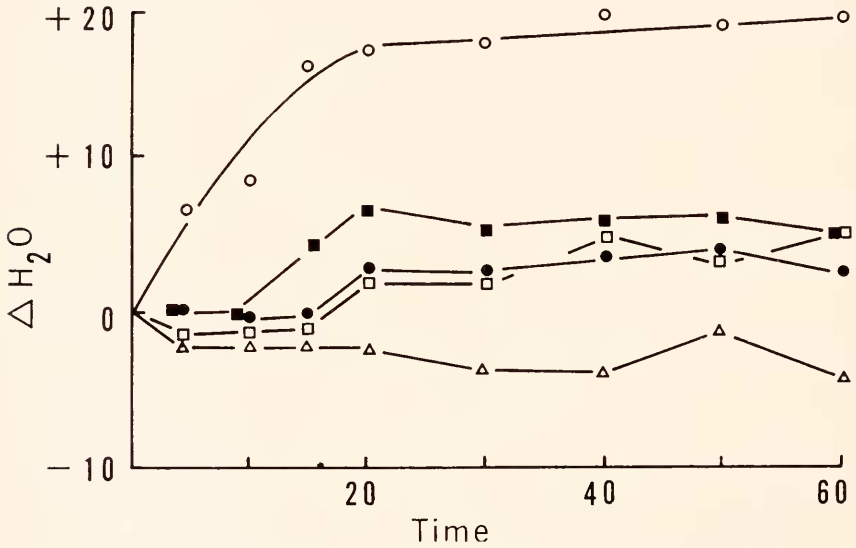

FIGURE 2. Percentage change in the worm water (ΔH_2O) of *Hymenolepis diminuta* incubated in 5 mM glucose in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Symbols as in Figure 1. Each point is the mean of 3 replicates.

RESULTS

The internal glucose concentration ($[G_i]$) of *H. diminuta* incubated in KRT for 60 min rose from an initial 4 mM to 12 mM, in terms of worm water. During this period, there was a net water influx representing a 5% increase in worm water, and a net decrease in worm glycogen. When incubated in 5 mM glucose in KRT for 60 min, the $[G_i]$ rose from 4.2 mM to 25.5 mM, and was accompanied by a 20% increase in worm water; under these conditions worm glycogen increased (Figs. 1 and 2, Table I). Glucose in the incubation medium decreased from 5.0 mM to 3.8 mM during the 60 min incubation. However, of the 12 μmoles removed from the incubation medium (10 ml at 1.2 μmoles/ml) by worms, only 5 μmoles

of free glucose was recovered in the ethanol extracts indicating that 60% of the absorbed glucose was metabolized.

When worms were incubated in 5 mM glucose in Na^+-free KRT (with choline as the replacement cation) for 60 min, the $[G_i]$ rose from 3.8 mM to 6 mM, while only 2–5% of the initial worm water was lost. Glucose in the incubation media increased from 4.98 mM to 5.25 mM after 60 min, and worm glycogen decreased (Figs. 1 and 2, Table I).

The steady state free glucose concentration of worms incubated in Cl^--free KRT (Cl^- replaced with CH_3COO^- or NO_3^-) with 5 mM glucose rose significantly above the glucose concentration of the media. With NO_3^- as the replacement anion the $[G_i]$ attained was approximately equal to that attained by worms incubated in glucose in KRT (27 mM and 25.5 mM, respectively), while in media with CH_3COO^- as the replacement anion the steady state $[G_i]$ was reduced (18 mM). There was a small net influx of water (5–6% of the original worm water) in worms incubated in glucose in Cl^--free KRT. Worms incubated in Cl^--free

TABLE I

Glycogen levels (mg/g wet wt) of Hymenolepis diminuta *before (control) and after a 60 min* in vitro *incubation in various media. Values are listed as means of 3 replicates ± S.E.*

Incubation medium	Glycogen
A. Control (none)	92.6 ± 5.6*
B. KRT	85.4 ± 4.8
C. 5 mM glucose in KRT	100.8 ± 3.9
D. 5 mM glucose in Na^+-free KRT**	86.4 ± 4.1
E. 5 mM glucose in Cl^--free KRT***	97.6 ± 4.6
F. 2 mM methionine in KRT	87.2 ± 4.1
G. 2 mM methionine in Na^+-free KRT**	87.8 ± 5.0
H. 2 mM methionine in Cl^--free KRT***	88.0 ± 4.5

* Statistical differences were determined using a one-way analysis of variance and Student-Newman-Keuls *a posteriori* test. The means were grouped as follows: (B, D, F, G, H) (A) (C, E); Each group of means is different from the other two groups at the 95% level of significance.

** Choline as the replacement cation.

*** CH_3COO^- as the replacement anion.

KRT with NO_3^- as the replacement anion removed a slightly greater amount of glucose than did worms in media with CH_3COO^- as the replacement anion; in both experiments 55–60% of the glucose removed from the media in 60 min was not recovered in the ethanol extracts, *i.e.*, it was metabolized. The glycogen levels of worms incubated in glucose in Cl^--free KRT increased (Figs. 1 and 2, Table I).

The method of Horn *et al.* (1946) was not sensitive enough to detect the small free methionine pool known to exist in *H. diminuta* (J. E. Simmons, Jr., unpublished), nor did this method reveal any changes in extractable methionine when worms were incubated in KRT for 60 min. However, when worms were incubated in 2 mM methionine in KRT, Na^+-free KRT (with choline as the replacement cation), or Cl^--free KRT (with either NO_3^- or CH_3COO^- as the replacement anion), increases in the free methionine of worms were easily detected, and worms were found to accumulate methionine in all media (Fig. 3). The internal methio-

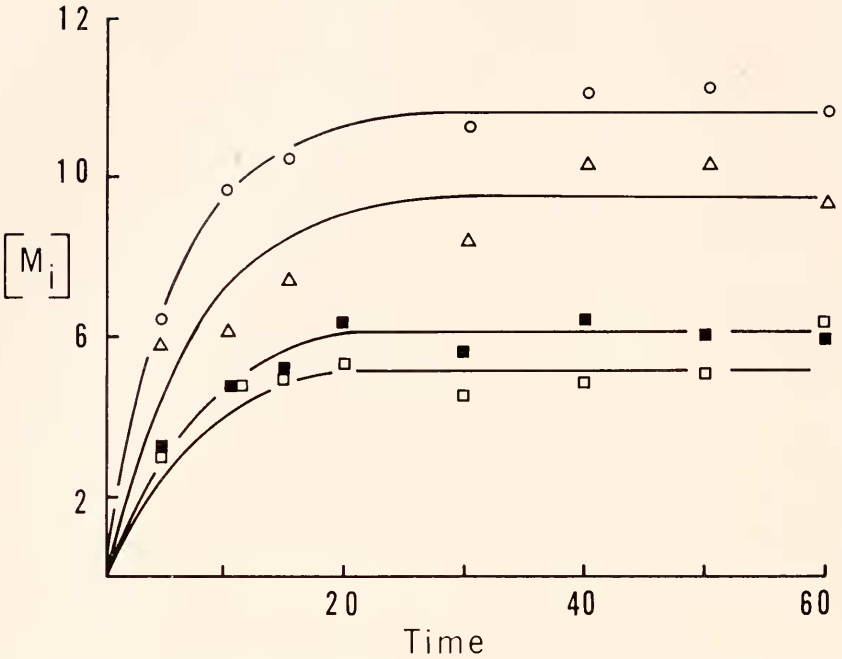

FIGURE 3. The internal methionine concentration ($[M_i]$, μmoles methionine/ml worm water) of *Hymenolepis diminuta* incubated in 2 mM methionine in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Open circles equal worms incubated in methionine in KRT; open triangles equal worms incubated in methionine in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in methionine in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3COO^- as the replacement anion. Each point is the mean of 3 replicates.

nine concentration ($[M_i]$) at a steady state was about 11.5 mM and 9.5 mM for worms incubated in 2 mM methionine in KRT and Na^+-free KRT, respectively. Although the steady state $[M_i]$ of worms in Na^+-free media appeared to be slightly depressed, the values for the $[M_i]$ of worms at 40, 50, and 60 min incubation in KRT and Na^+-free KRT were not significantly different ($P > 0.05$ by Student's t test). However, the steady state $[M_i]$ of worms incubated in 2 mM methionine in Cl^--free KRT (with either replacement anion) was significantly lower than that of worms incubated in methionine in KRT. $[M_i]$ values of worms incubated in NO_3^- and CH_3COO^--substituted media were not significantly different.

In KRT, there was a net water influx with about 5% increase in worm water; however, with the addition of 2 mM methionine, water effluxed from the worms regardless of the ionic composition of the media (Fig. 4). Water efflux from worms incubated in methionine in KRT or Na^+-free KRT was less than 5% of the original worm water, but in Cl^--free KRT, the water efflux rose as high as 10%. Glycogen levels of worms decreased when incubated in the absence of glucose (Table I). Methionine in the incubation media was not depleted to a significant extent in any experiment.

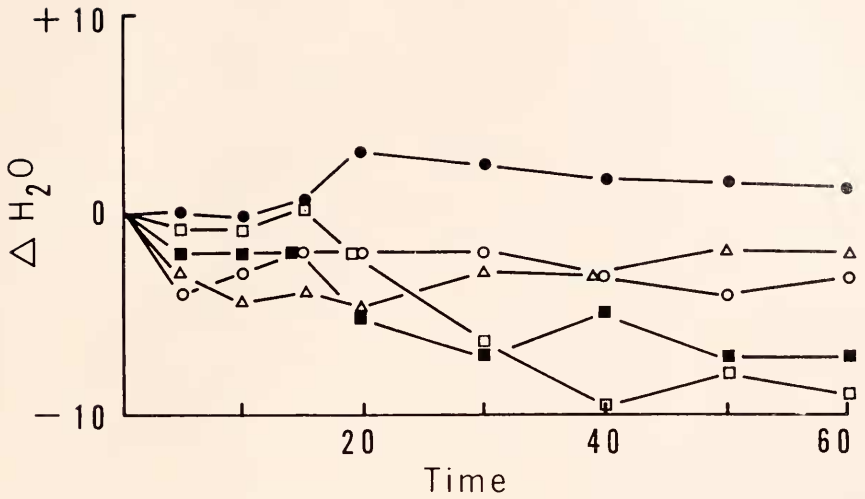

FIGURE 4. Percentage change in the worm water (ΔH_2O) of *Hymenolepis diminuta* incubated in 2 mM methionine in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Closed circles equal control (worms incubated in KRT only); open circles equal worms incubated in methionine in KRT; open triangles equal worms incubated in methionine in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in methionine in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3OO^- as the replacement anion. Each point is the mean of 3 replicates.

Glucose influx rates in *H. diminuta* were extremely sensitive to the external Na^+ concentration. Glucose influx was inhibited 96% in Na^+-free media (with choline as the replacement cation). Glucose influx rates were less sensitive to external Cl^- concentrations, and significant inhibition of glucose influx was obtained only at very low Cl^- concentrations (Table II). Methionine influx rates were unaffected by deletion of Na^+ or Cl^- from the media (Table III).

TABLE II

The effect of Na^+ and Cl^- deletion on the influx (μmoles/g ethanol extracted dry wt/2 min) of ^{14}C-glucose in Hymenolepis diminuta. *Values are listed as means of 3 replicates ± S.E.*

$[Na^+]$, mM	Choline as replacement cation	$[Cl^-]$, mM	CH_3COO^- as replacement anion	NO_3^- as replacement anion
0	0.05 ± 0.006*	0	8.61 ± 1.46**	7.38 ± 0.42*
5	0.65 ± 0.06	5	12.88 ± 0.96	—
10	1.26 ± 0.06	10	11.00 ± 0.73	7.01 ± 0.36
15	2.91 ± 0.16	25	13.89 ± 0.93	8.04 ± 0.29
25	3.96 ± 0.18	50	13.83 ± 1.22	8.31 ± 0.58
50	6.06 ± 0.23	75	14.39 ± 0.78	9.27 ± 0.59
75	7.86 ± 0.39	100	12.73 ± 0.88	9.78 ± 0.22
100	9.68 ± 0.67	130	14.58 ± 1.79	11.94 ± 0.18
154	11.15 ± 0.59			

* Glucose concentration = 1 mM
** Glucose concentration = 2.5 mM.

TABLE III

The effect of Na^+ and Cl^- deletion on the influx (μmoles/g ethanol extracted dry wt/2 min) of ^{14}C-methionine in Hymenolepis diminuta. *Values are listed as means of 3 replicates ± S.E.*

$[Na^+]$, mM	Choline replacement cation	$[Cl^-]$, mM	CH_3COO^- as replacement anion	NO_3^- as replacement anion
0	14.34 ± 0.89*	0	11.09 ± 0.22**	11.18 ± 0.58**
5	12.88 ± 0.38	5	10.80 ± 0.28	—
10	15.35 ± 0.60	10	—	10.98 ± 0.61
25	13.30 ± 0.40	15	11.52 ± 0.76	—
50	13.17 ± 0.78	25	11.83 ± 0.39	11.86 ± 0.51
100	16.30 ± 0.83	50	11.79 ± 0.54	12.13 ± 0.81
154	14.59 ± 0.64	75	12.26 ± 0.62	11.08 ± 0.72
		100	12.39 ± 1.06	11.82 ± 0.71
		130	12.00 ± 0.67	11.61 ± 0.68

* Methionine concentration = 2.5 mM.
** Methionine concentration = 2 mM.

DISCUSSION

Previous studies have shown that some tapeworms have Na^+-dependent glucose transport systems (see introduction), and *H. diminuta* is no exception. As previously shown by Dike and Read (1971), glucose influx in *H. diminuta* is inhibited almost completely in Na^+-free media, and more recent studies have shown that glucose and Na^+ fluxes in *H. diminuta* are coupled (Read, Stewart and Pappas, unpublished). It is, therefore, not surprising to find that *H. diminuta* does not accumulate glucose in Na^+-free media. Apparently, the lack of glucose accumulation is related to the Na^+-dependence of glucose fluxes, with the energy-coupling device for glucose accumulation being maintenance of a Na^+ gradient across the tapeworm tegument. It might be argued that glucose is not accumulated in Na^+-free media because the worms are not able to absorb glucose which would subsequently be used for energy production (Read, 1967); however, it should be noted that the worms produce glucose during incubation in Na^+-free media (*via* glycogenolysis), and also that worms accumulate methionine in the absence of an external energy source (*i.e.*, glucose). Therefore, the inhibition of glucose accumulation in Na^+-free media because of a lack of glucose as an energy source seems an improbable explanation.

When incubated in KRT only, the $[G_i]$ of worms increases significantly while glycogen levels decrease. (There is little doubt that this increase in tissue glucose is a direct consequence of glycogenolysis, since glucose is the only glycogenic monosaccharide [Read, 1967], and since worms degrade glycogen when incubated in the absence of external glucose.) We might expect similar results from worms incubated in glucose in Na^+-free KRT since, under these conditions, no glucose enters the worms. Although glycogen levels in worms incubated in glucose in Na^+-free KRT decrease, there is no concomitant increase in tissue glucose. This apparent discrepancy might be explained by the observation that the glucose concentration of the Na^+-free media increases during incubation with worms, the additional glucose coming from glycogenolysis and subsequent glucose efflux from

the worms. Such a "trans" effect from a reversed Na^+ difference (gradient) also occurs in the tapeworm *C. verticillatum*, as shown by Pappas and Read (1972). These data support the hypothesis that glucose fluxes in *H. diminuta* are controlled, in large part, by the prevailing Na^+ difference.

Although pigeon erythrocytes contain at least 3 specific amino acid transport systems, one of which is sensitive to both Na^+ and Cl^- (Imler and Vidaver, 1972), no animal cell, to our knowledge, has been shown to contain a single glucose transport system which is sensitive to both anions and cations. *H. diminuta* may be the exception since glucose influx is inhibited in both Na^+- and Cl^--free media. No data are available which suggest a possible mechanism for this Cl^--sensitivity. However, since replacement of Cl^- with NO_3^- or CH_3COO^- yields similar results (38% and 41% inhibition, respectively) and since methionine influx is not inhibited in Cl^--free media, it appears that the effects of Cl^- deletion are rather specific. Although these data are suggestive of an anion requirement for glucose influx, further studies are needed to clarify this point.

H. diminuta accumulated glucose in Cl^--free media, but the steady state $[G_i]$ of worms is reduced when CH_3COO^- is used to replace deleted Cl^-. Since replacement of Cl^- with either NO_3^- or CH_3COO^- has similar results on glucose influx, we may speculate that replacement of Cl^- with CH_3COO^- increases the efflux of glucose from *H. diminuta*. This should be examined experimentally. However, it is clear the glucose influx and accumulation in *H. diminuta* do not display an absolute requirement for Cl^-, as they do for Na^+.

As originally reported by Read *et al.* (1963), methionine influx in *H. diminuta* is not Na^+-sensitive. Similar results were obtained by Uglem (unpublished) with lysine, glutamate and leucine influxes in *H. diminuta*, and Pappas *et al.* (1973b) showed that methionine, phenylalanine and arginine influxes into *T. crassiceps* larvae are not Na^+-sensitive. Methionine accumulation by *H. diminuta* is also Na^+-insensitive, as is methionine and phenylalanine accumulation by *T. crassiceps* larvae (Pappas *et al.*, 1973b). Unlike glucose influx and accumulation, methionine influx and accumulation in *H. diminuta* and *T. crassiceps* larvae are not dependent upon Na^+ in the external medium. A similar situation may exist in the Acanthocephala, since Uglem and Read (1973) found that leucine is accumulated by *Moniliformis dubius* in Na^+-free media.

Deletion of Cl^- and its replacement with NO_3^- or CH_3COO^- has no effect on methionine influx, however, the steady state $[M_i]$ of worms incubated in 2 mM methionine in Cl^--free media is reduced, compared to worms incubated with methionine in KRT. It appears that either amino acid influx and accumulation are not Cl^--sensitive, or that both NO_3^- and CH_3COO^- will replace Cl^- if this system is anion sensitive. In pigeon erythrocytes, glycine influx occurs through a system which cotransports Na^+ but which also must bind an anion for amino acid translocation. F^-, NO_3^-, HCO_2^-, SCN^-, and I^- will replace Cl^- as the anion cofactor, although they are less efficient than Cl^-, while CH_3COO^-, $CH_3CH_2(OH)COO^-$ (lactate), and larger anions will not replace Cl^- (Imler and Vidaver, 1972). Methionine influx in *H. diminuta* apparently occurs through a system quite different than that found in other anion and/or cation-requiring systems.

As stated above, the data regarding glucose influx and accumulation are consistent with Na^+-gradient hypothesis as originally proposed by Crane (1962, 1965).

However, methionine influx and accumulation do not depend upon the maintenance of either a Na^+ or Cl^- difference across the cell membrane. That is, the maintenance of an ion difference does not appear to be involved in energy-coupling for methionine accumulation, as the data suggest it to be for glucose accumulation. Therefore, we may infer that the energy necessary for methionine accumulation must be derived from some other source. Several studies have shown that the potential energy available from the Na^+ difference across cell membranes is sufficient to account for amino acid and glucose accumulation in some mammalian intestinal preparations and pigeon erythrocytes, however, in other systems (chick intestine and ascites tumor cells), the Na^+- gradient does not appear to provide sufficient potential energy for solute influx and accumulation (see Gibb and Ebby, 1972, and Johnstone, 1972, and references therein), and it has been suggested that additional energy is derived from the maintenance of K^+ difference or from ATP directly. No studies are available which deal with energy requirements of solute influx and accumulation in *H. diminuta,* but it appears that the energy for methionine accumulation must be derived from some source other than Na^+ or Cl^- concentration differences across the cell membranes.

For the preceding discussion we have made the tenuous, but necessary, assumption that water associated with *H. diminuta* (as determined from [wet wt]–[dry wt]) exists in a free state within this tapeworm and that solutes are in "true solution." In a recent symposium (Hazelwood, 1973) the physical and chemical state of water and solutes within living cells was discussed at length and, from data presented in these papers, it is apparent that neither of our assumptions are completely valid. However, at the present time an accurate estimation of "bound" *versus* "unbound" water and solutes cannot be made. This situation is complicated by the water fluxes associated with solute accumulation by *H. diminuta.* Although these fluxes are generally small ($< 5\%$ of the original worm water), glucose accumulation by *H. diminuta* is accompanied by a 20% increase in worm water, and methionine accumulation in Cl^--free media is accompanied by a 10% loss of worm water. Like the physico-chemical state of water already present in the worm tissues, the true state of the water influxing during glucose accumulation is uncertain. In addition, many tapeworm species, including *H. diminuta,* appear to have the capacity to regulate their water volume (Read, Douglas and Simmons, 1959; Read and Simmons, 1963; Smyth, 1969; DeRycke, 1972), although the exact mechanism of this water regulation is unknown. It is apparent, therefore, that additional studies are necessary, not only to determine the physicochemical state of solutes and water within *H. diminuta,* but also to determine the osmoregulatory capabilities of tapeworms and the significance of the water fluxes associated with solute accumulation.

Summary

When incubated in 5 mM glucose in a buffered Krebs-Ringer saline for 60 min, *Hymenolepis diminuta* accumulated glucose to a steady state concentration of 25.5 mM; in Cl^--free saline, with either NO_3^- or CH_3COO^- as the replacement anion, glucose was accumulated by worms. *H. diminuta* incubated in 5 mM glucose in Na^+-free media, with choline as the replacement cation, did not accumulate glucose. Short term glucose influx (2 min) in *H. diminuta* was inhibited 96% in Na^+-free

media, and approximately 40% in Cl^--free media. The data support the hypothesis that glucose influx and accumulation in *H. diminuta* occurs through a Na^+-dependent system similar to that proposed by the "ion-gradient hypothesis." The data suggest further that glucose influx in *H. diminuta* may be anion-sensitive as well.

H. diminuta accumulated methionine when incubated in 2 mM methionine in normal saline, Na^+-free media, or Cl^--free media, and short term methionine influx (2 min) in *H. diminuta* was unaffected by Na^+ or Cl^- deletion. Methionine influx and accumulation are not dependent on anion or cation differences (gradients) across the cell membranes and the energy necessary for solute accumulation must be derived from some source other than maintenance of these ion-differences.

Solute accumulation in *H. diminuta* was accompanied by significant water fluxes in some instances. Since the physical state of water and solutes within worms and the worms osmoregulatory capacities are unknown, the significance of these water fluxes is uncertain.

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